Tannase activity by lactic acid bacteria isolated from grape must and wine

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Abstract

We examined a range of oenological lactic acid bacteria species and reference strains for their potential to degrade tannins. Bacterial tannase activity was checked by a spectrophotometric and a visual reading method. None of the strains belonging to the oenological species of the genus *Lactobacillus*, *Leuconostoc*, *Oenococcus* or *Pediococcus* were tannase producers, with the exception of *Lactobacillus plantarum*. All the *L. plantarum* strains analyzed were positive for tannase activity and their identities were reconfirmed by a *L. plantarum* PCR–specific assay or by sequencing the 16S rDNA. Tannase activity could be considered an important criterion for the selection of malolactic starter cultures since it might confer advantages in the winemaking process by reducing astringency and haze in wine.

Keyworks: Tannase, Wine, Lactic acid bacteria, *Lactobacillus plantarum*, wine haze

1. Introduction

Tannin acyl hydrolase (E.C. 3. 1. 1. 20), commonly called tannase, catalyzes the hydrolysis of ester bonds in hydrolyzable tannins such as tannic acid, thereby releasing glucose and gallic acid (Lekha and Lonsane, 1997). Tannins are defined as naturally occurring water–soluble polyphenols of varying molecular weight depending on the bonds possessed with proteins and polysaccharides (cellulose and pectin). Tannins are widespread in the plant kingdom, are found in leaves, fruits, bark and wood.

Tannase finds widespread application in food and beverage processing. At the moment most of the commercial applications of tannase are in the manufacturing of instant tea –where it is used to eliminate water insoluble precipitates–, wine, beer and coffee–flavored sotf drinks. Other important application of tannase in the food industry, is its use as substrate for the chemical synthesis of pyrogalol or ester galates, which are used as preservatives. Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used as antioxidant in fats and oils, as well as in beverages (Lekha and Lonsane, 1997).

In the case of wines, tannase hydrolyzed chlorogenic acid to caffeic acid and quinic acid, which favorably influences taste (Lekha and Lonsane, 1997). Tannase has been used along with laccase for the treatment of grape juice and grape musts to remove phenolic substances for chemical stabilization of the beverage. Moreover, fifty percent of the colour of the wine is due to the presence of tannins; however, if these compounds are oxidized to quinones by contact with the air, they could form an undesirable turbidity, which causes severe quality problems. The use of tannase has been proposed as the best solution to this problem (Aguilar and Gutierrez–Sánchez, 2001).

Tannase can be obtained from plant, animal and microbial sources. The most important source to obtain the enzyme is by microbial way, because the produced enzymes are more stable than similar ones obtained from other sources (Bhat et al., 1998). It has long been known that several fungal species such as *Aspergillus* spp. (Banerjee and Mondal, 2001) and *Penicillum* spp. (Rajakumar and Nandy, 1983) are capable of producing large amounts of tannase. Over the past decade, many bacterial species have also been reported to produce tannase. These include *Streptococcus gallolyticus* (Osawa et al., 1995a), *Lonepinella koalarum* (Osawa et al., 1995b), *Bacillus licheniformis* (Mondal and Pati, 2000), and several lactobacilli species (Osawa et al., 2000). However, there is no report describing the presence of tannin–degrading bacteria in the wine–related microbiota in spite of the undoubted interest of this enzymatic activity in the wine–making process.

The aim of this study was to examine the occurrence of tannase activity in several strains of LAB isolated from Spanish grape musts and wines.

2. Materials and methods

2.1. *Strains and growth conditions*

Pure cultures of wine LAB control strains were provided by the Spanish Type Culture Collection (CECT). A total of 78 LAB were obtained from the bacterial culture collection of the Instituto de Fermentaciones Industriales (IFI), CSIC, Spain. These strains were originally isolated from must grape or wine of different wine–producing areas of Spain and classified by using biochemical tests (Moreno–Arribas et al. 2003).

Strains of *O. oeni* were grown on medium for *Leuconostoc oenos* (MLO medium) (Caspritz and Radler, 1983) supplemented with 10% tomato juice. The other LAB tested were grown in MRS broth (Difco, France). All bacteria were incubated at 30 ºC in a 5% CO_2 atmosphere.

2.2. Bacterial DNA extraction

Chromosomal DNA from selected LAB strains was isolated using the following protocol. Briefly, the LAB strains were grown at 30 ºC for 48 h in 10 ml of MRS broth. The cells were pelleted by centrifugation and resuspended in 600 µl of TE (10 mM Tris–ClH pH 8.0, 1 mM EDTA) solution containing 10 mg/ml of lysozyme (Sigma, Germany). The cells were lysed by adding 70 µl of 10% SDS (w/v) and 10 µl of proteinase K (20 mg/ml) (Sigma, Germany). Crude DNA preparation was purified by performing two phenol:chloroform:isoamyl alcohol (25:24:1) and one chloroform:isoamyl alcohol (24:1) extractions. Chromosomal DNA was precipitated by adding two volumes of cold ethanol. The precipitated DNA was washed with 70% ethanol and left to air dry. The DNA pellet was dissolved in an appropriate volume of TE buffer.

2.3. Lactobacillus plantarum identification based on PCR

The PCR–specific reaction of *Lactobacillus plantarum* were performed using the specific primers LbPl1 (5´–AATTGAGGCAGCTGGCCA–3´) and LbPl2 (5´–GATTACGGGAGTCCAAGC–3´) described by Quere et al. (1997). These primers

amplified a 250 pb fragment in all the *L. plantarum* strains tested. This DNA fragment revealed no homology to any known sequences contained in standard databases. PCR reaction was performed in 0.2 ml microcentrifuge tubes in a total volume of 25 µl containing 1µl of template DNA (aprox. 100 ng), 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, primer LbPl1 (1µM), primer LbPl2 (1µM) and 1 U of Ampli*Taq* DNA polimerase. The reaction was performed in a GeneAmp PCR System 2400 (Perkin Elmer) using the following cycling parameters: initial 5 min denaturation at 94 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 50 ºC and 30 sec at 72 ºC. Amplified products were analyzed by 2% agarose gel electrophoresis in TAE buffer (Sambrook et al., 1989). The gel was stained with ethidium bromide and the bands were visualized under UV illumination.

2.4. PCR amplification of 16S rDNA

16S rDNAs were PCR amplified using the eubacterial universal pair of primers 63f (5´–CAGGCCTAACACATGCAAGTC–3´) and 1387r (5´–GGGCGGWGTGTACAAGGC–3´) (W = A or T) previously described (Marchesi et al., 1998). The 63f and 1387r primer combination generates an amplified product of 1.3 kb. PCR reaction was performed as described above. PCR cycling parameters were: initial 5 min denaturation at 94 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 50 ºC and 1:30 min at 72 ºC. Amplified products were resolved on a 0.7% agarose gel. The amplifications products were purified on QIAquick spin Columns (Quiagen) for direct sequencing.

2.5. DNA sequencing

DNA sequencing was carried out by using an Abi Prism 377^{TM} DNA sequencer (Applied Biosystems, Inc). Sequence similarity searches were carried out using Basic local alignement search (BLAST) (Altschul et al., 1997) on the EMBL/GenBank databases.

2.6. Tannase activity

Tannase activity of the isolates was tested following the spectrophotometric and visual reading methods described by Osawa and Walsh (1993). Briefly, fresh cultures on MRS or MLO agar plates were harvested with sterile cotton swabs and suspended in 1 ml of substrate medium (pH 5.0) containing 33mM NaH_2PO_4 and 20mM methylgallate (Fluka Chemie, Germany) to prepare a dense suspension (at least equivalent to a no. 3 McFarland turbidity standard). The substrate medium was then incubated aerobically at 37 °C for 24 h. After incubation, the sample was alkalinized with an equal amount of saturated $NaHCO_3$ solution (pH 8.6) and exposed to the atmosphere at room temperature (23 °C) for 1 h. Green to brown coloration of the medium was judged as a positive indicator of tannase activity in the visual reading method. In the spectrophotometric method, 1ml of the suspension was removed, centrifuged and the supernatant was read at A_{440} in a spectrometer (S–22 UV/vis spectrophotometer, Boeco, Germany).

3. Results and discussion

A number of microorganisms –including bacteria, fungi, and yeasts–have been reported to produce tannase. Extensive screening studies have been conducted to select potent cultures for tannase production. Bacterial species frequently isolated from wine were not included in previous studies. In this work, a wide range of wine lactic acid bacterial species was examined for tannase activity. In our study, several strains, including type strains, belonging to *L. mesenteroides* (11 strains), *Oenococcus oeni* (41 strains), pediococci (4 strains), *Lactobacillus buchneri* (9 strains), *L. fermentum* (1 strain), *L. fructivorans* (1 strain), *L. hilgardii* (8 strains) species were analysed for tannase activity. The tannase–positive *Lactobacillus plantarum* ATCC 14917 strain and tannase–negative *L. brevis* ATCC 8287 (CECT 4669) strain (Osawa et al., 2000) were used as reference strains in our screening.

Table 1 shows the number of positive strains of the total number of strains investigated. The tannase–negative strains by the visual method showed absorbance readings at 440 nm below 0.2 and the reaction media presented an light yellow colour (data not shown). However, absorbance values up to 0.3 were considered by Osawa and Walsh (1993) as negative for tannase production in two *Streptococcus bovis* strains analyzed. As could be observed from Table 1 tannase positive strains, including the positive control strain, showed a brownish colour of the media and presented absorvance values ranging from 0.716 to 0.980. In the study of Osawa and Walsh (1993) tannase positive enterobacterial strains gave absorbance values higher than 2.0; however, they considered also as positive absorvance values above 0.5.

In our study, identical results were obtained by using the spectrophotometric and the visual reading method in order to identify tannase–producing bacterial strains.

Therefore, as described previously (Osawa and Walsh, 1993), it might be concluded that the visual reading method is a simple method for detection of bacterial tannase, it does not require sophisticated analytic equipment and it can be incorporated into a conventional test system for bacterial identification.

As deduced from Table 1, it is noteworthy that tannase–producing strains only belongs to the *Lactobacillus plantarum* species. Previously, Osawa et al. (2000) isolated lactobacilli with tannase activity from human feces and fermented foods. All the tannase–producing isolates belong to the *L. plantarum*, *L. paraplantarum* and *L. pentosus*. They examined for tannase activity a range of 14 different *Lactobacillus* species obtained from culture collections. All the strains belonging to these 14 different *Lactobacillus* species were negative for tannase activity.

Bacterial species frequently isolated from wine were not included in previous studies. This is the first report on the tannase activity from strains belonging to *Leuconostoc*, *Oenococcus* or *Pediococcus* genus. In the same way, there are not tannase–activity studies of strains belonging to *L. buchneri*, *L. fermentum*, *L. fructivorans* and *L. hilgardii* species, which are frequently isolated from wines.

In this study, all the strains presumptively classified as *L. plantarum* were positive for tannase–activity. We used a previously described PCR–based method (Quere at al., 1997) for the specific identification of *L. plantarum*. The method is designed to amplify a 250 pb DNA fragment *L. plantarum* specific. We performed this PCR assay on total DNA extracted from the presumptively identified *L. plantarum* strains (*L. plantarum* ATCC 14917, and BIFI–31, 34, 35, 38, 39, 40, 41, 71, 72 and 73). Figure 1 showed the results of the PCR amplification. As expected, all the strains analysed gave a 250 bp DNA band specific of this species. The PCR results confirmed the classification of these strains as members of the *L. plantarum* species.

Surprisingly, besides the *L. plantarum* isolates another LAB strain gave a positive reaction for tannase activity. This strain, BIFI–28, was presumptively identified as *Oenococcus oeni*. To confirm the taxonomical identity of this strain, a 1.3 kb DNA fragment coding for the 16S rRNA was amplified. The bacterial isolate identified as being positive for tannase activity was then identified using sequence data from the first 500 bp of the 16S rRNA gene. The sequence obtained was identified by database comparison (BLAST search) using the GenBank nucleotide database (Altschul et al., 1997). This isolate contained a sequence identical with that of *L. plantarum* strains included in the databases. Therefore, the BIFI–28 isolate needs to be reclassified as *L. plantarum* BIFI–28 (Table 1).

From these results it may be concluded that all the *L. plantarum* isolates analyzed were the only tannase–producers strains. This result concurs with those of Osawa et al. (2000), which showed that tannase activity is common in *L. plantarum* strains. They postulated that this enzymatic property have an ecological advantage for this specie, as it is often associated with fermentations of plant materials.

During winemaking process, lactic acid bacteria are responsible of the malolactic fermentation (MLF). Since the beginning of the 1980s, commercial starter cultures for the induction of MLF have been available, consisting of strains of *O. oeni*, *L. plantarum* and *L. hilgardii* as single–or multiple–strain preparations. Tannase activity could be considered an criterion for the selection of malolactic starter cultures (Buckenhüskes, 1993) since it might represent several advantages in winemaking. First of all, tannins are well known to interact with proteins. Clear beverages are generally intended to remain clear until they are purchased and consumed. The most frequent cause of haze in wine results from protein–polyphenol interaction (Siebert, 1999). During vinification, a part of the soluble grape proteins is precipitated via interaction

with tannins. These precipitates are generated in a natural way when the beverage is cooled at temperatures lower than 4 ºC, and if this are removed chemically (employing bentonite), a great amount of aromatic compounds can be eliminated. Tannase hydrolyses the esters bonds from polyphenols, avoiding their polymerization, giving a wine with a high content of aromatic compounds and appropriate colour, increasing its quality (Aguilar and Gutierrez–Sánchez, 2001).

Besides, hydrolysable tannins extracted from the oak barrel during wine ageing, are the main responsible of the wine astringency (Robichaud and Noble, 1990). Thus interactions of tannins with proteins also occur in the oral cavity where salivary proteins precipitate, giving rise to the mouth sensation of astringency. During storage and ageing of wine, astringency decreases as these large molecules precipitated and the wine´s capacity to bind proteins fell. In a similar manner, the astringency could decrease without any precipitate by the use of tannase that hydrolyses tannins present in the wine.

In summary, this is the first study reporting the occurrence of lactobacilli capable of degrading hydrolyzable tannin in wine. *L. plantarum* was the only species showing tannase activity. None of the other lactobacilli species or species from the genus *Leuconostoc, Oenococcus* or *Pediococcus* were tannase–producers. Due to the potential beneficial effects of tannase during winemaking, this activity could be considered as an important criterion for the selection of malolactic bacterial starters.

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Figure captions

Figure 1. PCR amplification of a specific–DNA fragment from *L. plantarum*. Chromosomal DNA from the strains previously classified as *L. plantarum* were used for PCR amplification with oligonucleotides LbPl1 and LbPl2. (1) *L. plantarum* ATCC 14917; (2) BIFI–31; (3) BIFI–34; (4) BIFI–35; (5) BIFI–38; (6) BIFI–39; (7) BIFI–40; (8) BIFI–41; (9) BIFI–71; (10) BIFI–72; and (11) BIFI–73. Products were subject to agarose gel electrophoresis and stained with ethidium bromide. Left lane, 50–bp Ladder. Numbers indicate some of the molecular sizes (in bp).

Table 1
Tannase-producing LAB strains isolated from grape must and wine

Name as collected	Strain	Source	Tannase		Taxon confirmed by PCR[c] or sequencing[d]
			A_{440}[a]	Color[b]	
Lactobacillus plantarum	ATCC[e] 14917[T]	Pickled cabbage	0.846	+	*L. plantarum*[c]
	BIFI[f]-31	Wine	0.716	+	*L. plantarum*[c]
	BIFI-34	Wine	0.825	+	*L. plantarum*[c]
	BIFI-35	Wine	0.957	+	*L. plantarum*[c]
	BIFI-38	Wine	0.768	+	*L. plantarum*[c]
	BIFI-39	Wine	0.861	+	*L. plantarum*[c]
	BIFI-40	Wine	0.736	+	*L. plantarum*[c]
	BIFI-41	Wine	0.980	+	*L. plantarum*[c]
	BIFI-71	Wine	0.811	+	*L. plantarum*[c]
	BIFI-72	Wine	0.829	+	*L. plantarum*[c]
	BIFI-73	Wine	0.845	+	*L. plantarum*[c]
Oenococcus oeni	BIFI-28	Wine	0.863	+	*L. plantarum*[d]

[a] A_{440}, absorbance reading at 440 nm
[b] +, green to brown tinging of the medium
[c] PCR assay as described by Quere et al.(1997)
[d] DNA sequencing of the 16S rDNA amplified using primers 63f and 1387r (see section 2.4)
[e] ATCC, American Type Culture Collection
[f] BIFI, Bacterial culture collection from the Instituto de Fermentaciones Industriales